Optical Microscopy

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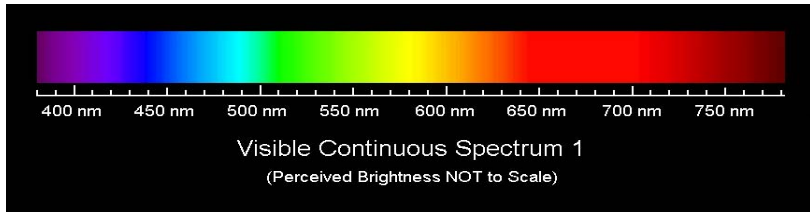
Syllabus:
Optics & Microscopy

1.)Optics & Objective Lens
- a) Geometric Optics
  - Light, Refraction, Refractive Index, Dispersion
  - The eye
  - Image formation $1/i + 1/o = 1/f$
  - Magnification
  - Aberrations: chromatic & spherical
  - NA, light collection
  - Oil and Water lenses
  - Markings on an Objective lens
- b) Physical Optics
  - Diffraction
  - Information Transfer (thro the lens)
  - NA, Resolution

2) Microscopy
- a) Illumination & Brightfield Microscopy
  - Critical Illumination
  - Kohler Illumination
  - Field Stop (diaphragm)
  - Aperture Stopa)
- b) Phase Microscopy
  - Intensity Vs Phase Object
  - Diffraction & the Zeroth Ordar
  - Quarter Wave Plate
- c) Fluorescence Microscopy
  - Fluorescence
  - Epi illumination
  - Excitation Filters, Barrier Filters and Dichroic Mirrors
  - Mercury Arc lamps
- d) Confocal Microscopy
  - The basis of a confocal microscope is a pin hole
  - Using a pin hole means a different image detector is required
  - Using a pin hole means the illumination is scanned over the object
  - Spinning Disk Confocal
- e) Some Practical Aspects
  - How to set up Kohler
  - Cleaning
  - How to care for lens
  - How to change the arc lamp
  - How to choose the best objective for fluorescence
  - Which fluorphors work best.

Geometric Optics



Visible Spectrum 400nm-800nm
500nm = 0.5um = 5000mm

The job of a lens is to collect light from each point on the object and to refract it to a corresponding point on the image

Notice that not all the light from the object is collected. This results in a loss of information in the image

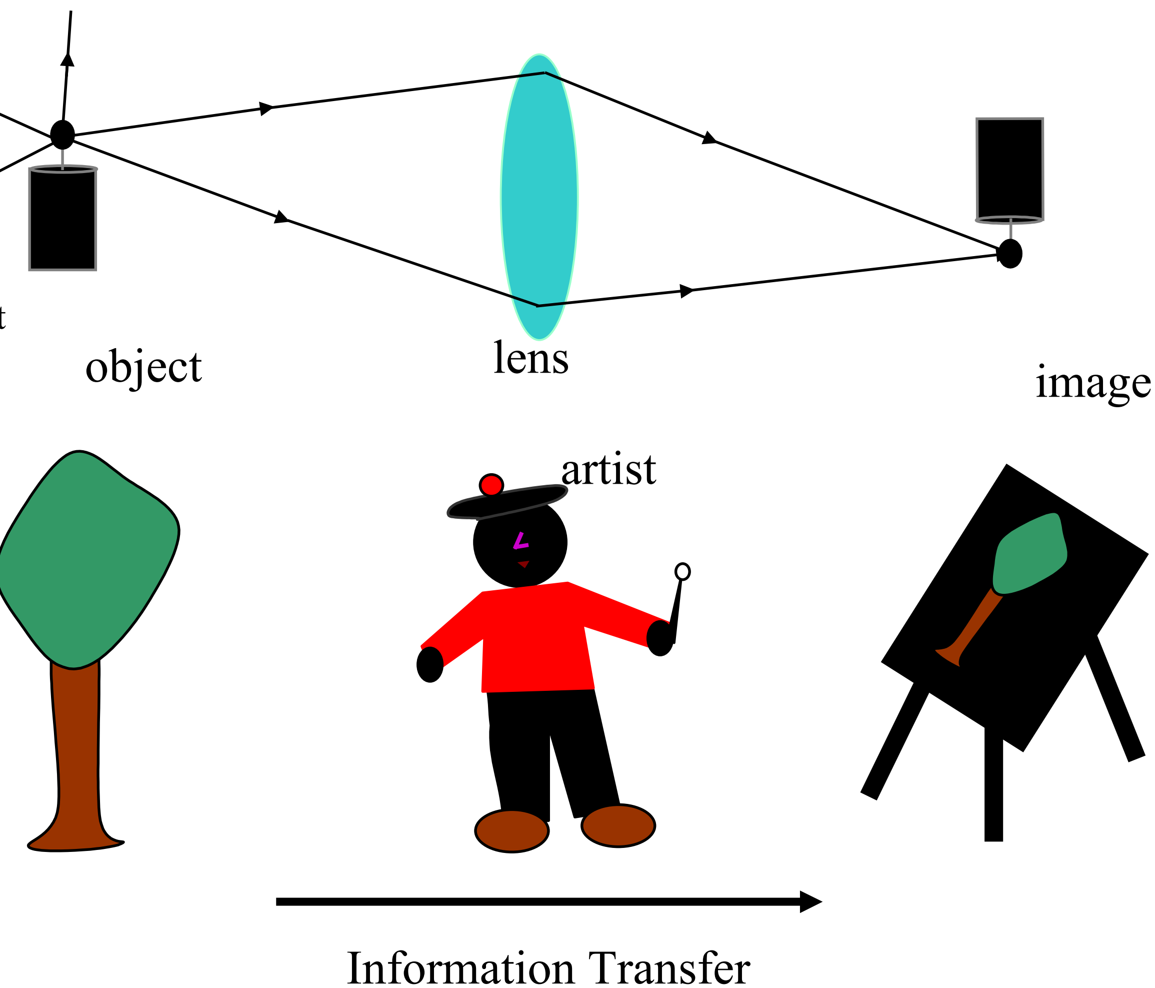
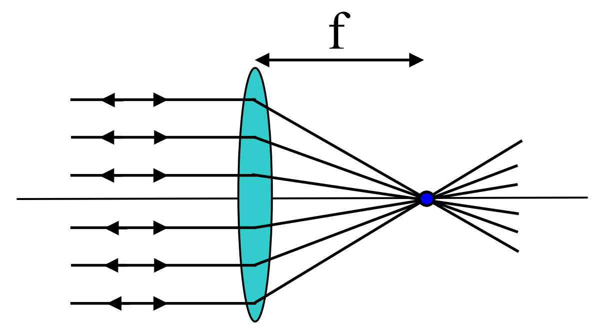
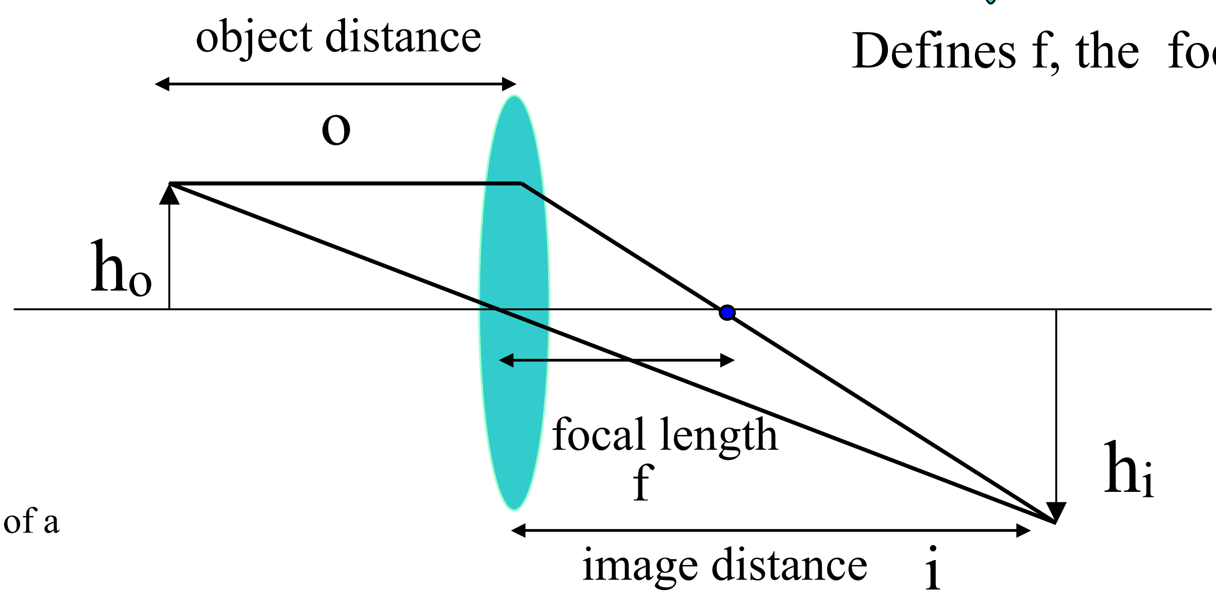


Image Formation

Rays parallel to the optical axis are refracted thro the focal point of the lens



Defines f, the focal length

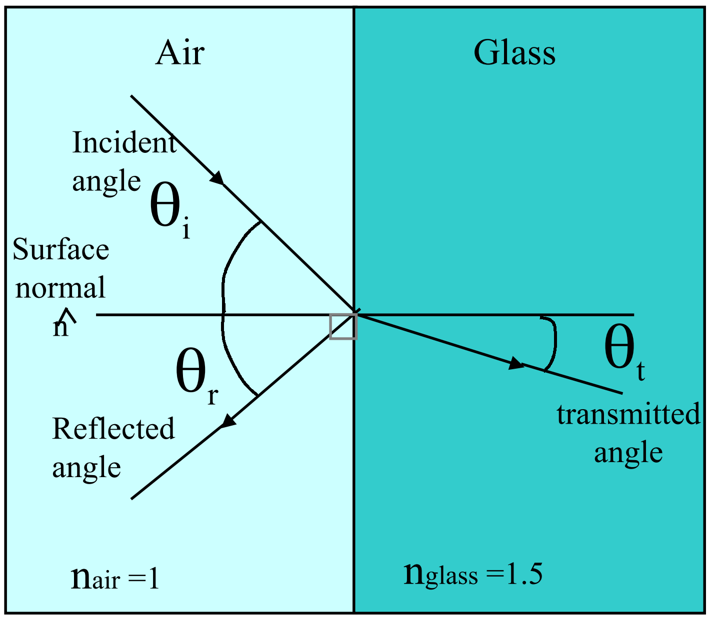


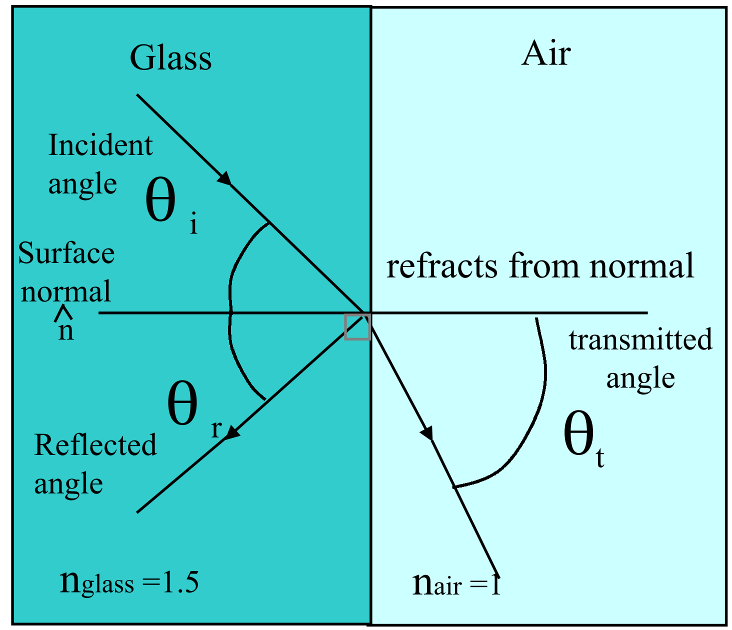
Rays thro the center of a lens are unrefracted

$$1/i + 1/o = 1/f$$

$$M = h_i / h_o$$

Reflection and Refraction





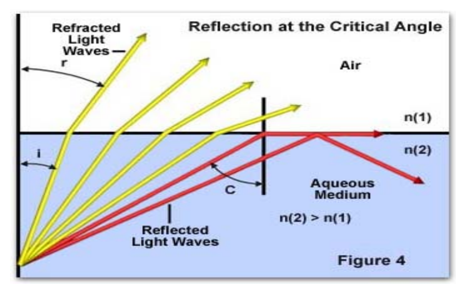
<u>Refractive Index</u>

$n_{medium} = C / V_{medium}$

The direction of a ray of light changes as the refractive index it propagates in changes

<u>Snell's Law</u>:

$$n_1 \sin\theta_1 = n_2 \sin \theta_2$$
$$\theta_i = \theta_r$$
$$\sin \theta_t = n_i / n_t \sin\theta_i$$

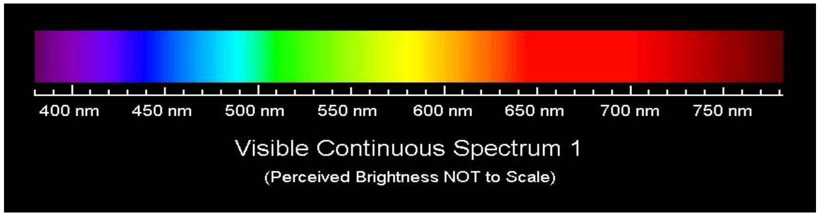


<u>Total Internal Reflection</u>

occurs when $\theta_t = 90^o$ at an incident angle $\theta_{critical}$

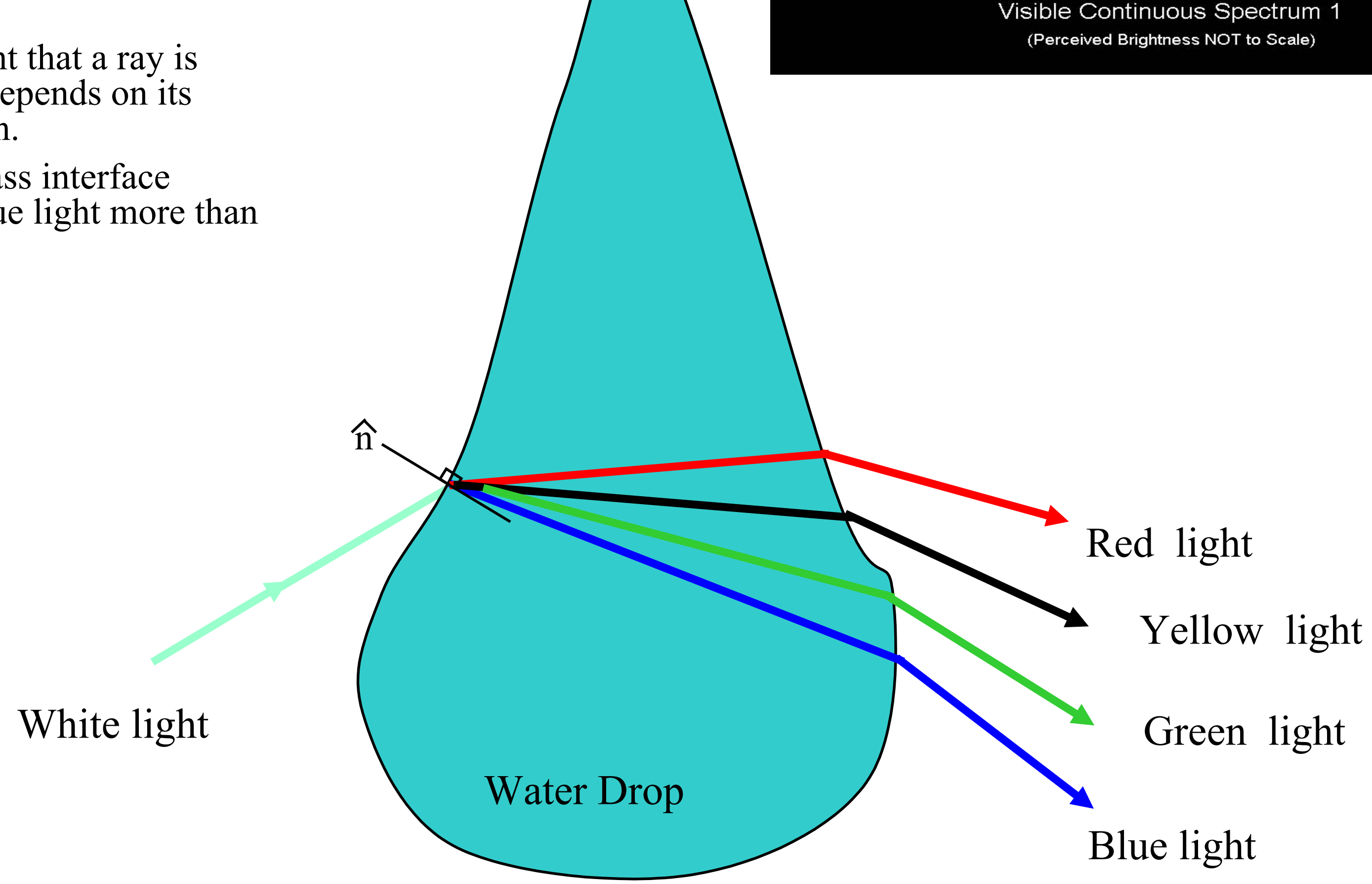
$\sin\theta_{critical} = n_{air} / n_{glass}$

Dispersion



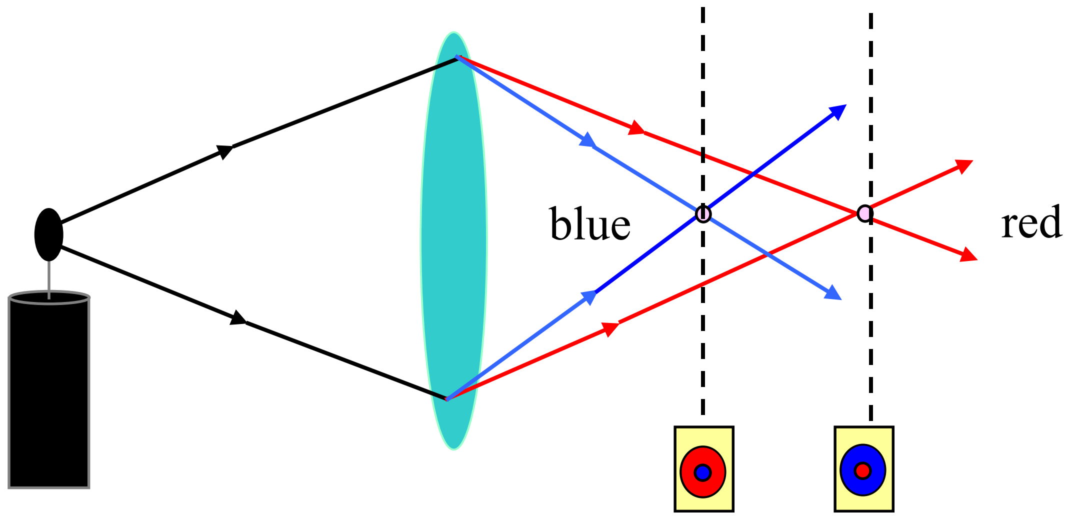
The amount that a ray is refracted depends on its wavelength.

The air-glass interface refracts blue light more than red light.

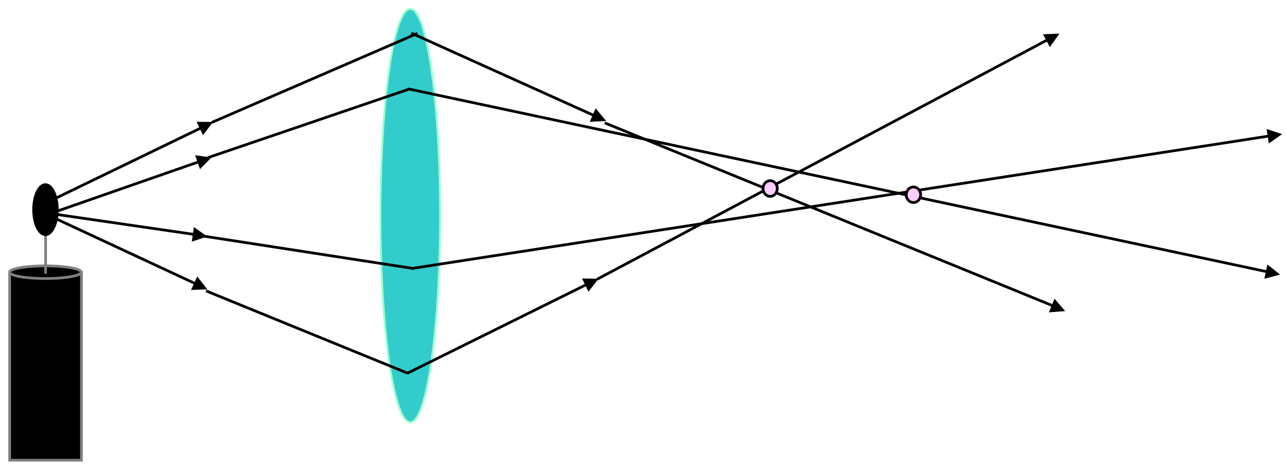


Aberrations

Chromatic Aberration



Spherical Aberration



There are many effects that cause a lens to produce multiple points of focus in the image even though the light was collected from one point on the object.

These situations are termed aberrations and result in blurred, poor quality images.

Aberrations & Compound Objective Lens

Aberrations can be corrected by adding specialized optical elements to the lens.

Such corrections make lens bulky, expensive and more specialized in their function.

<u>Corrections</u>

Plan / Plano - Flat Field

Achromat / Achro - Red/Blue Chromatic

Fluorite / Fluor - 2 color Chromatic & Spherical

Apochromats / Apo - Red/Green/Blue Chromatic

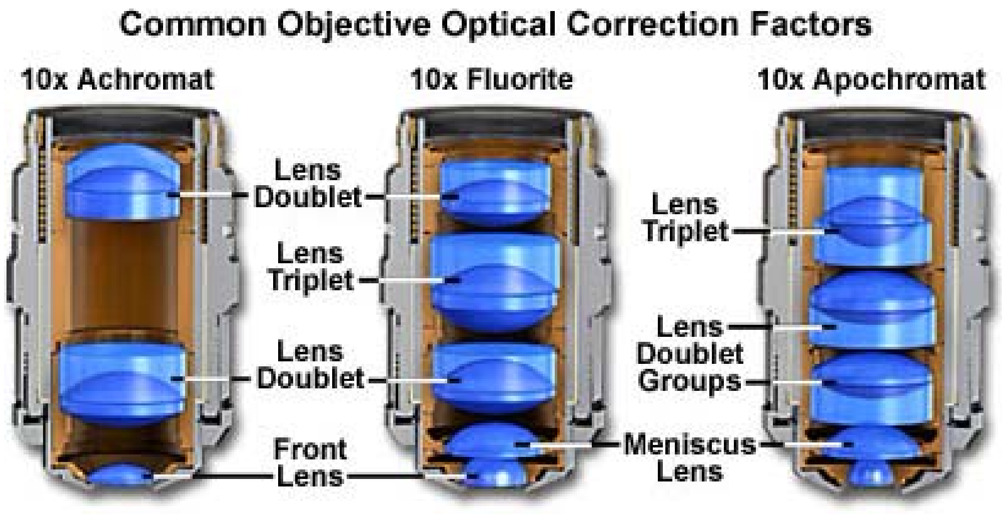
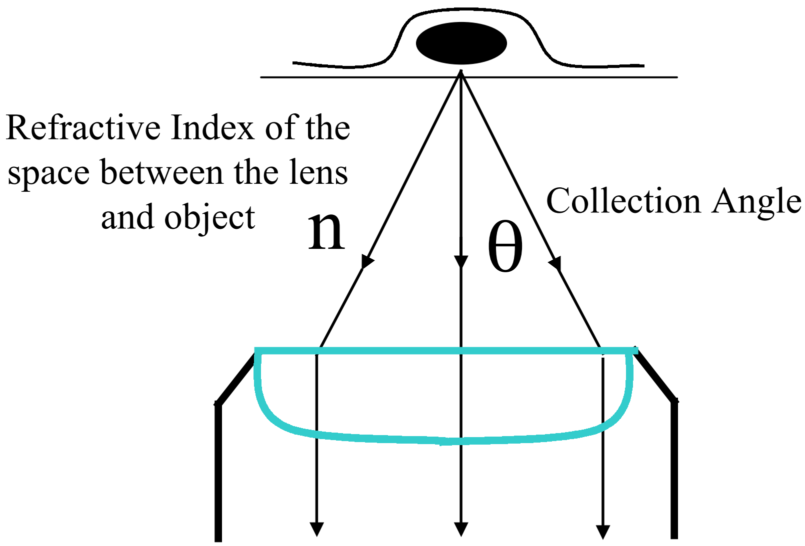


Figure 2

Figure 1

Numerical Aperture



60x Plan Apochromat Objective

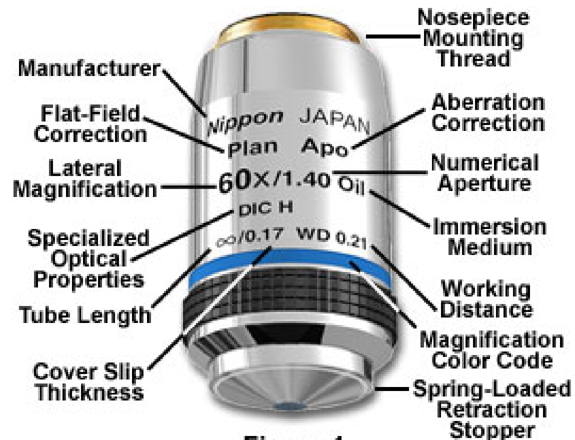


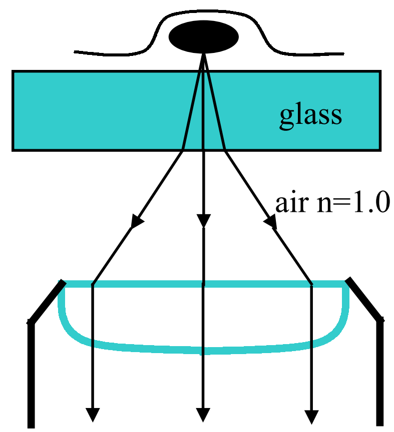
Figure 1

$$NA = n \sin\theta$$

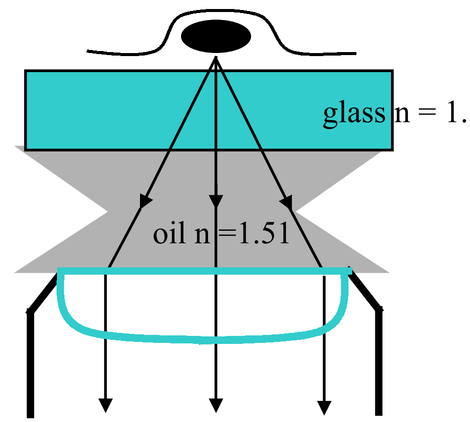
As the numerical aperture, NA, is increased, both the amount of light and the amount of information collected from the object increases.

However, as the NA increases so does a lens susceptibility to aberrations.

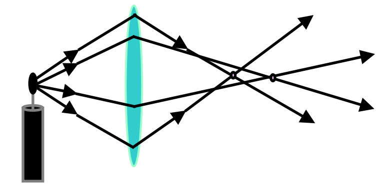
Dry Objective

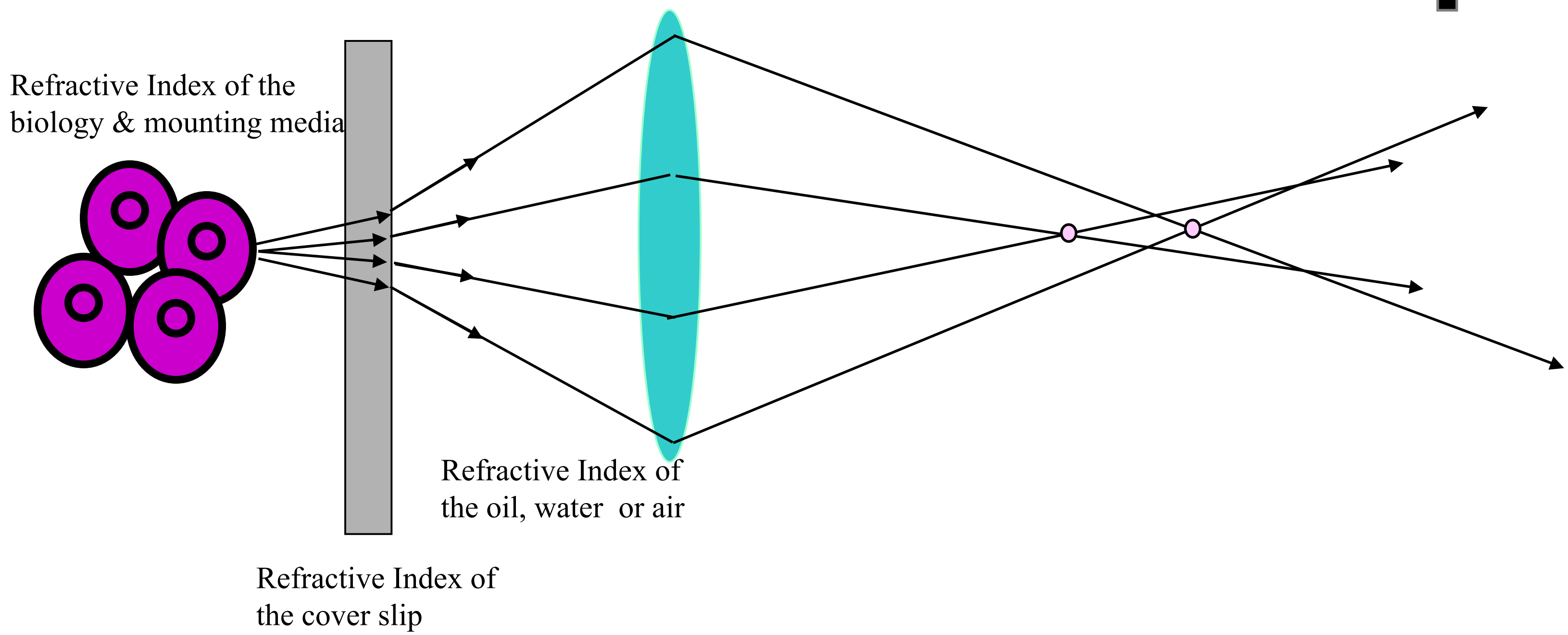


Oil Objective



Spherical Aberration: Cover Glasses, Thick Samples & Correction Collars





Due to refraction at the cover glass-air boundary, rays collected at the perimeter of the lens appear to emanate from a point closer to the lens than rays collected thro the center of the lens. Thus, rays at the perimeter get focused further away from the lens than rays thro the perimeter, even though all the rays came from a single object point and even though the lens is corrected for spherical aberration.

This type of aberration is also commonly referred to as spherical aberration, and is the reason air lens must be corrected for such.

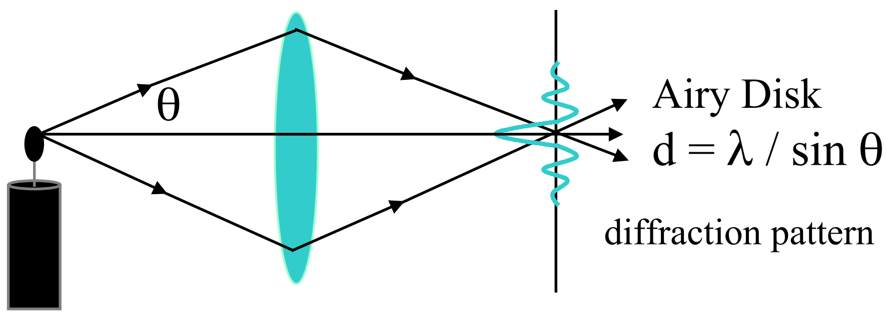
Refraction mismatch due to the biology being imaging or the mounting media can also severely effect image quality when collecting from deep within thick samples.

The severity of this problem increases with the numerical aperture, NA, of the objective lens.

Physical Optics

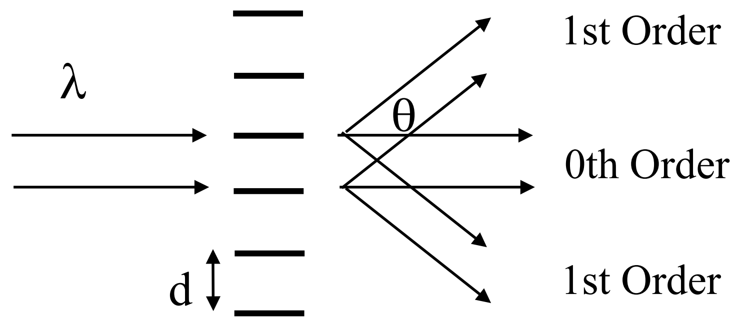
Diffraction Limited

Even without geometric aberrations a lens is unable to reproduce a point in the image for every point in the object. It produces a "diffraction limited" spot called the Airy disk. For the artist this is determined by the size of the brush and for a lens it is the wavelength of the light.

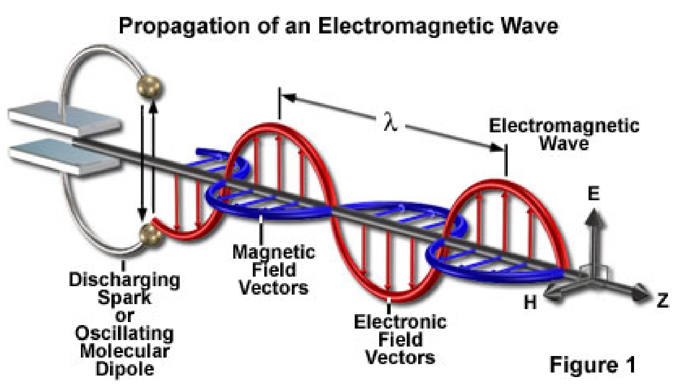


Fraunhofer Diffraction

$\lambda = d \sin \theta$

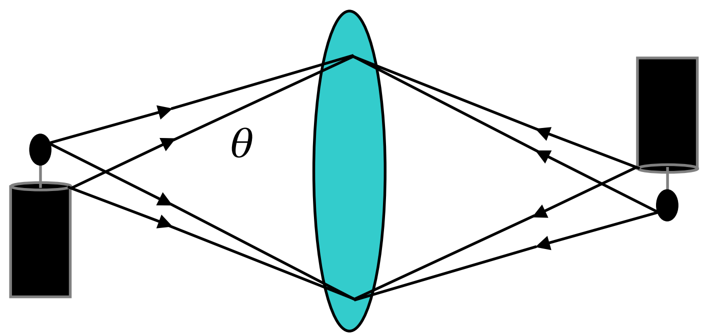


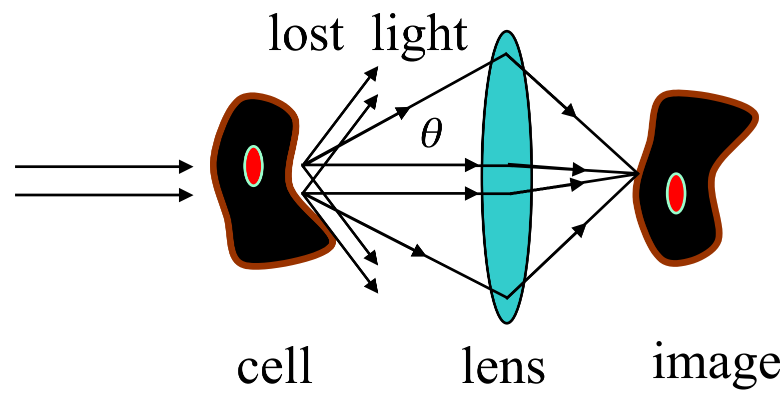
Wave nature of light

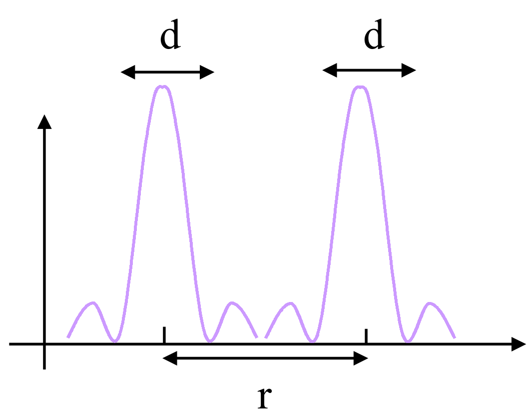


Resolution

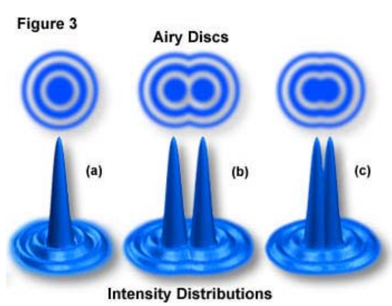
Resolution : The distance between 2 points on the object that are separable (resolved) in the image







Rayleigh Criterion
resolution r = d/2 = λ / 2 sin θ



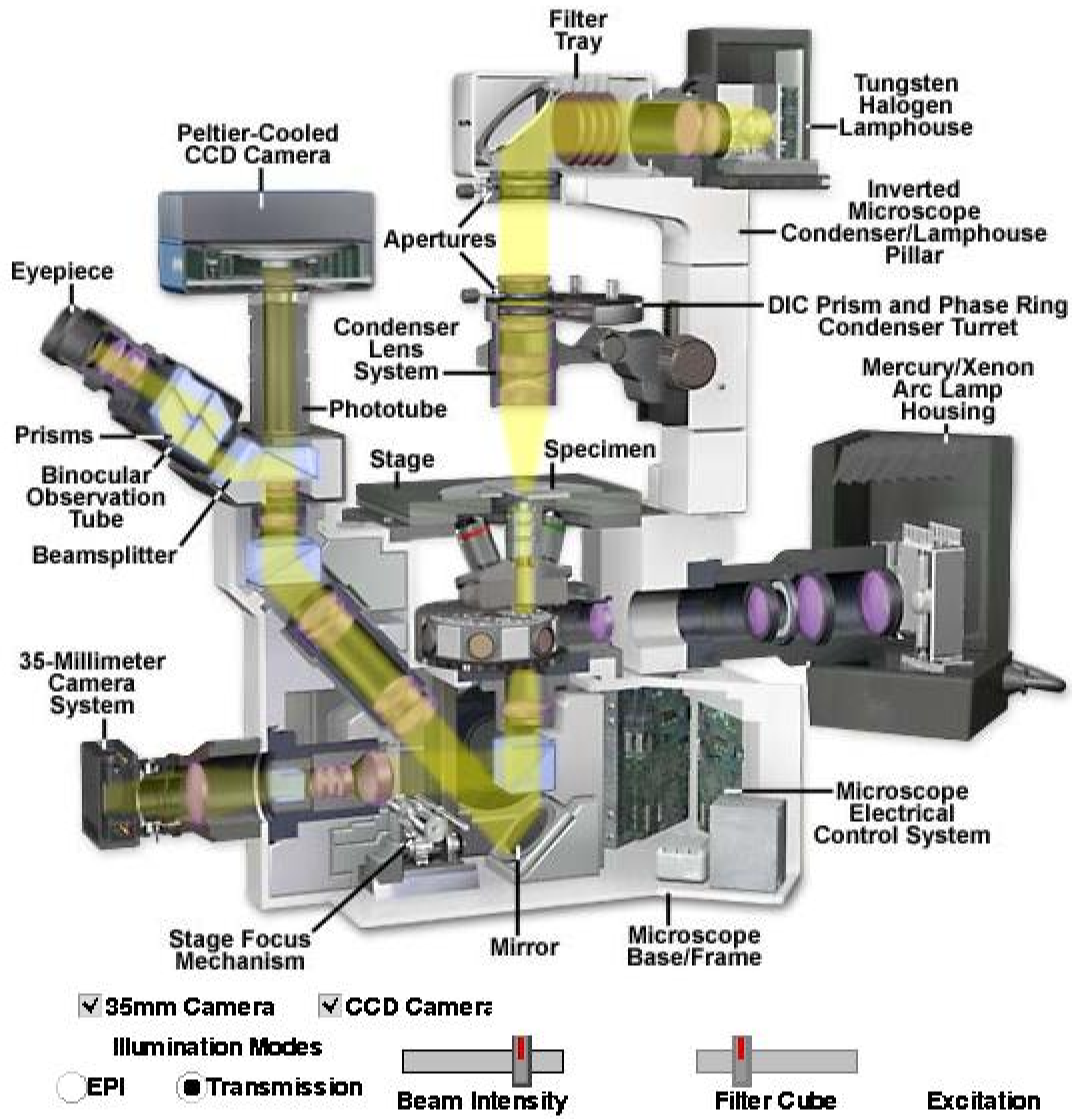
- very fine structure in the object diffracts light which is not collected by the lens and not represented in the image
- the finest details represent in the image have a separation (resolution) given by the NA of the lens

r ~ d = λ / sin θ

Resolution r = λ/ 2NA

Microscopy

Illumination
Phase Microscopy
Fluorescence Microscopy
Confocal Microscopy

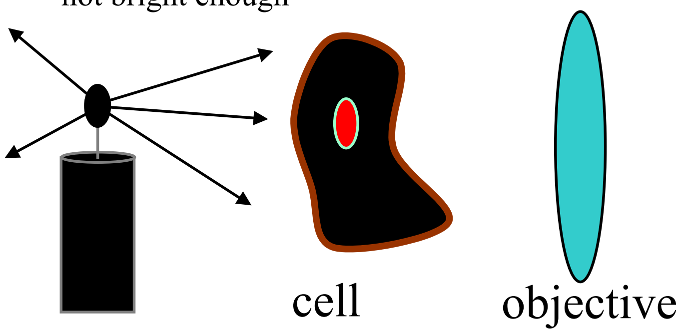


Illumination

Task : Brightly and evenly illuminate the object with light that matches the NA of the objective lens being used

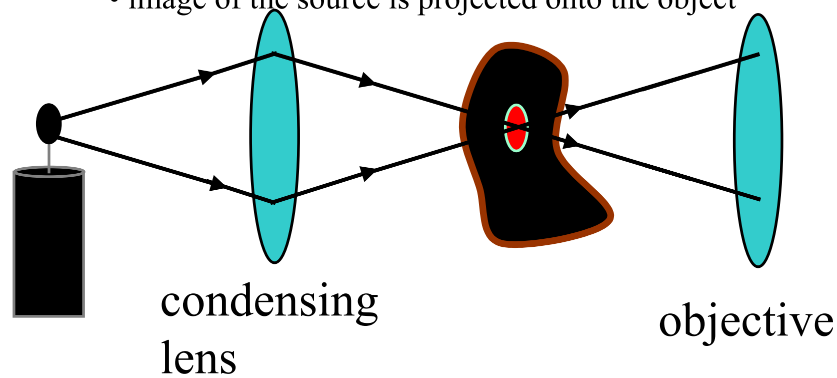
Direct Illumination

• not bright enough

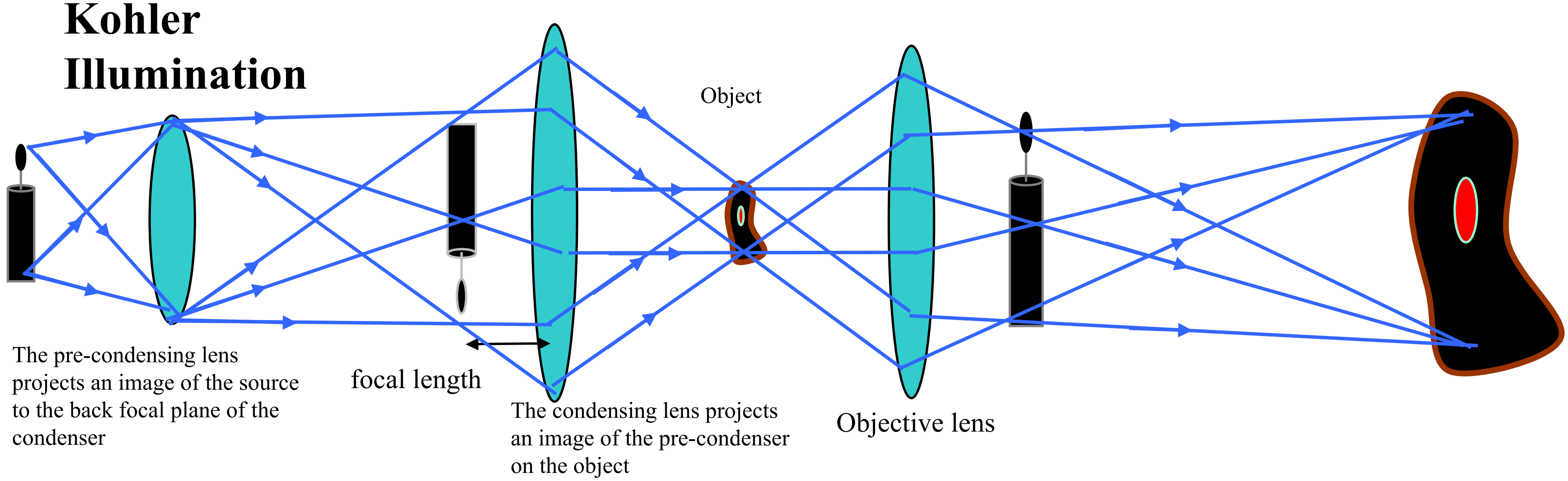


Critical Illumination

• image of the source is projected onto the object



Kohler Illumination



Field and Aperture Stop

The Field diagram is placed at the pre-condensing lens. Its image is projected onto the object plane of the microscope by the condensing lens. It controls the size of the illumination field of the object.

The Aperture or the Iris diaphragm is positioned at the back focal plane of the condenser lens and controls the NA of the cone of light the condenser produces.

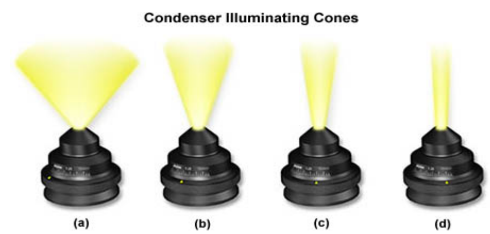
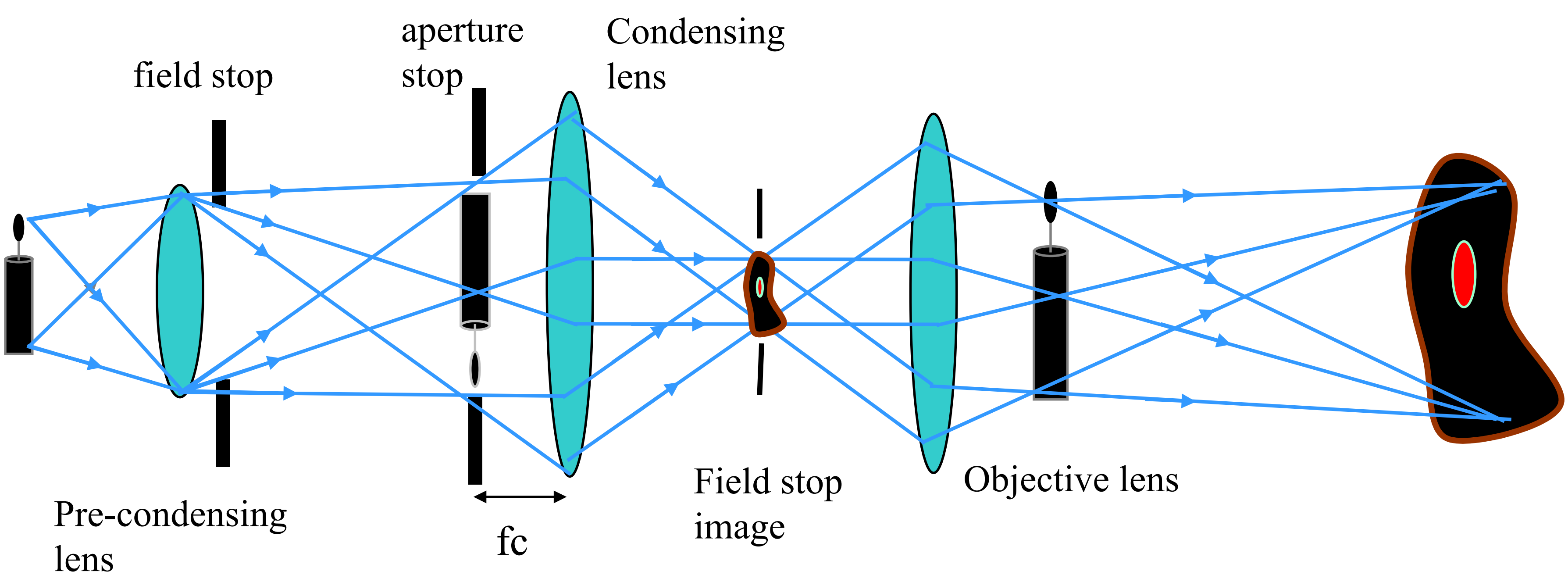


Figure 3

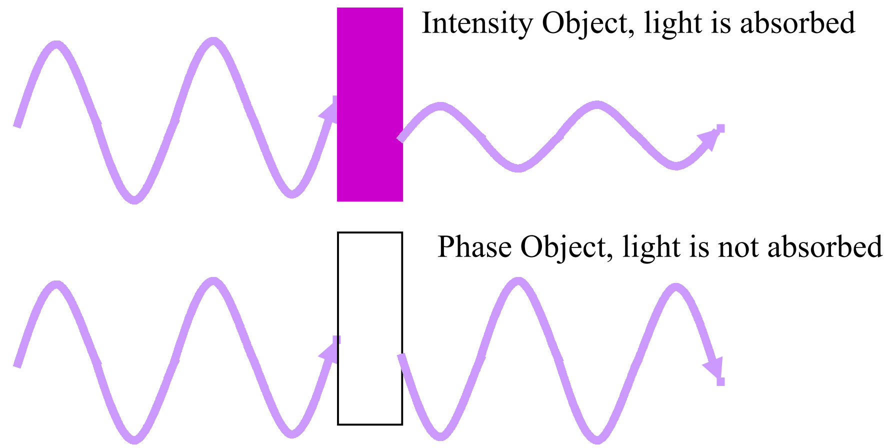
Kohler Illumination

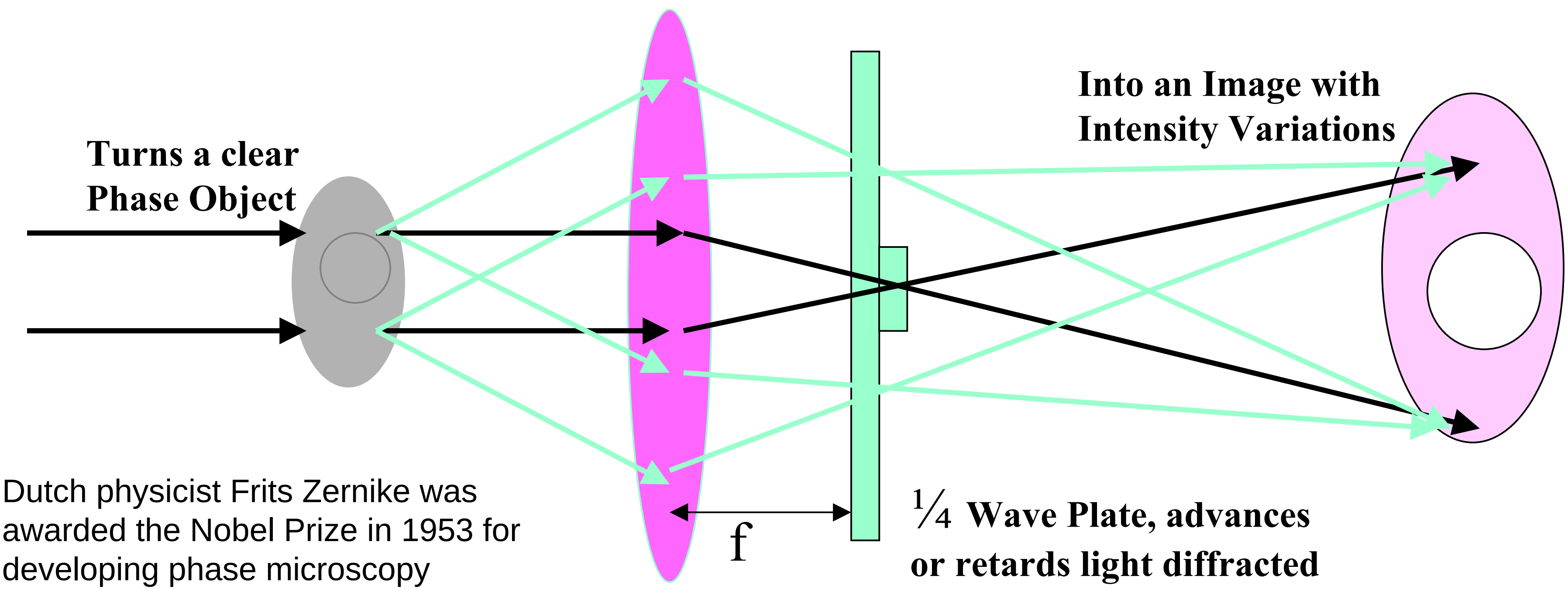


Phase Microscopy

• When light hits an object, some of the light is diffracted and some is not. The undiffracted light (Zeroth Order) determines the background intensity of the image while the diffracted light carries the information about the object to the image.
• In phase microscopy the phase of the zeroth order is advanced or retarded by ¼ wavelength before it recombines with the higher orders to create the image.
• In dark field microscopy the zeroth order light is simply blocked from the image.

Intensity Vs Phase Object

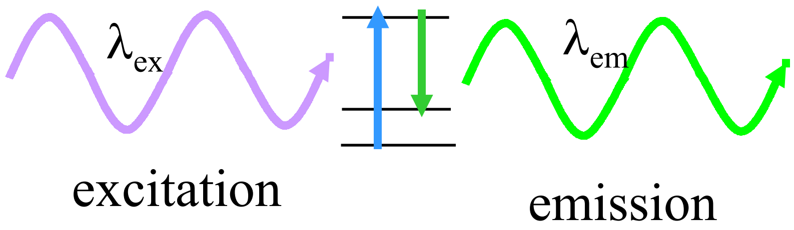




Dutch physicist Frits Zernike was awarded the Nobel Prize in 1953 for developing phase microscopy

Fluorescence Microscopy

Fluorescence



$E = hf = hc / \lambda, \qquad \lambda_{em} > \lambda_{ex}$

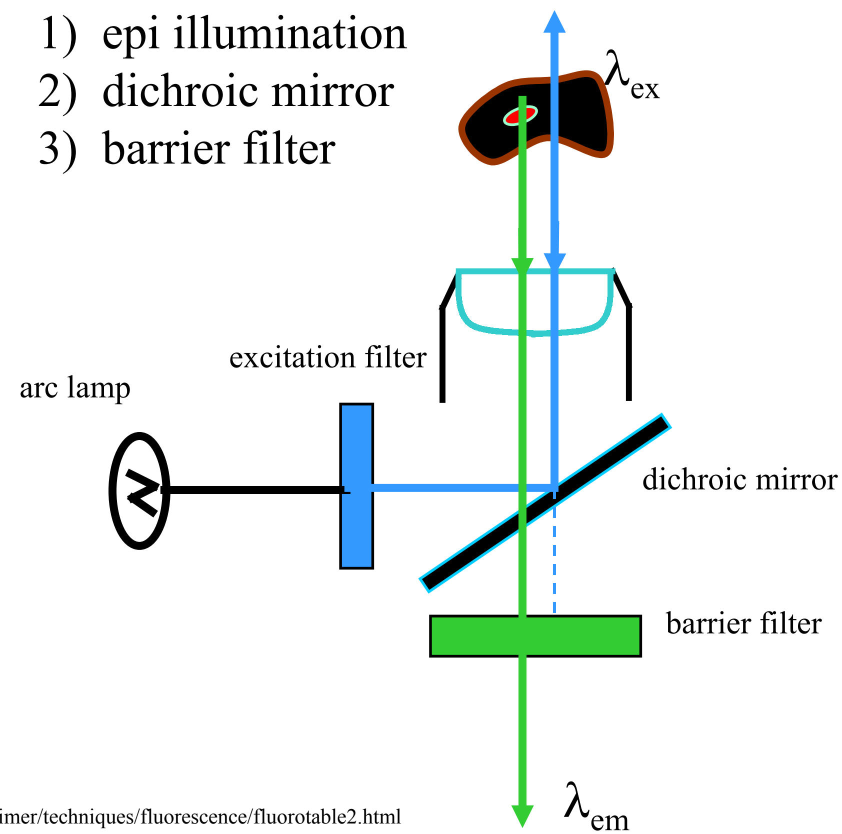
Some fluorphors

dye	Ex/ Em nm
Alexa 350	346/442
dapi	360/460
e-gfp	489/508
gfp (wild type)	395/509
flouroscein	495/518
alexa 488	346/442
sytox green	504/523
eosin	524/544
rhodamine	580/590
cy3.5	581/596
alexa568	578/603
texas red	595/615
alexa633	632/647
to-pro3	642/661
cy5	633/700

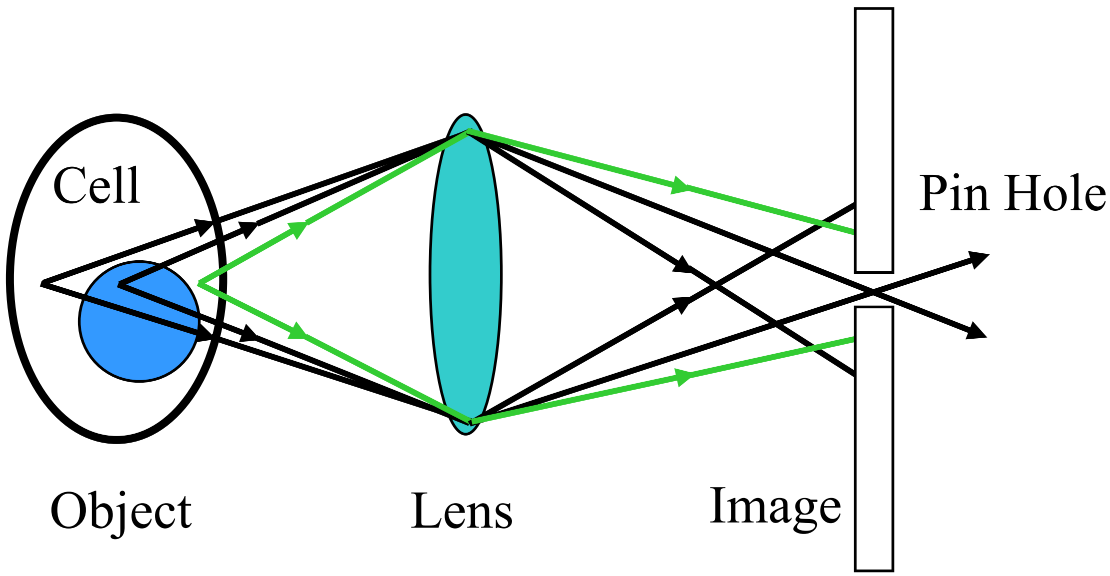
http://www.microscopy.fsu.edu/primer/techniques/fluorescence/fluorotable2.html
http://microscopy.bio-rad.com/fluorescence/fluorophoradata.htm

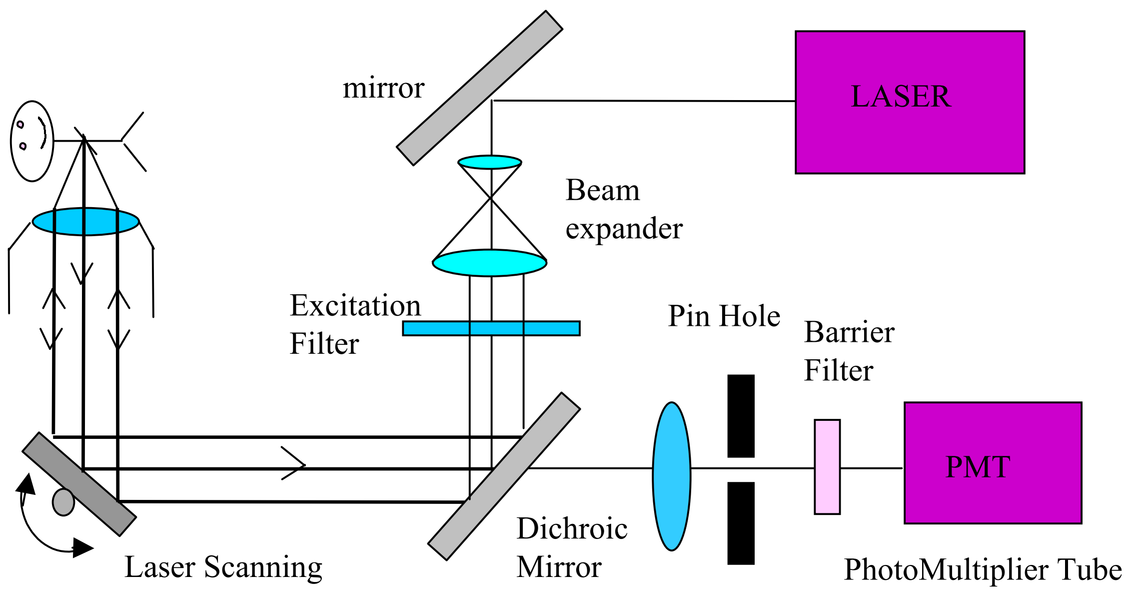
The trick is to filter out λ_{ex} which is done in three steps.

1) epi illumination
2) dichroic mirror
3) barrier filter



Confocal Microscopy





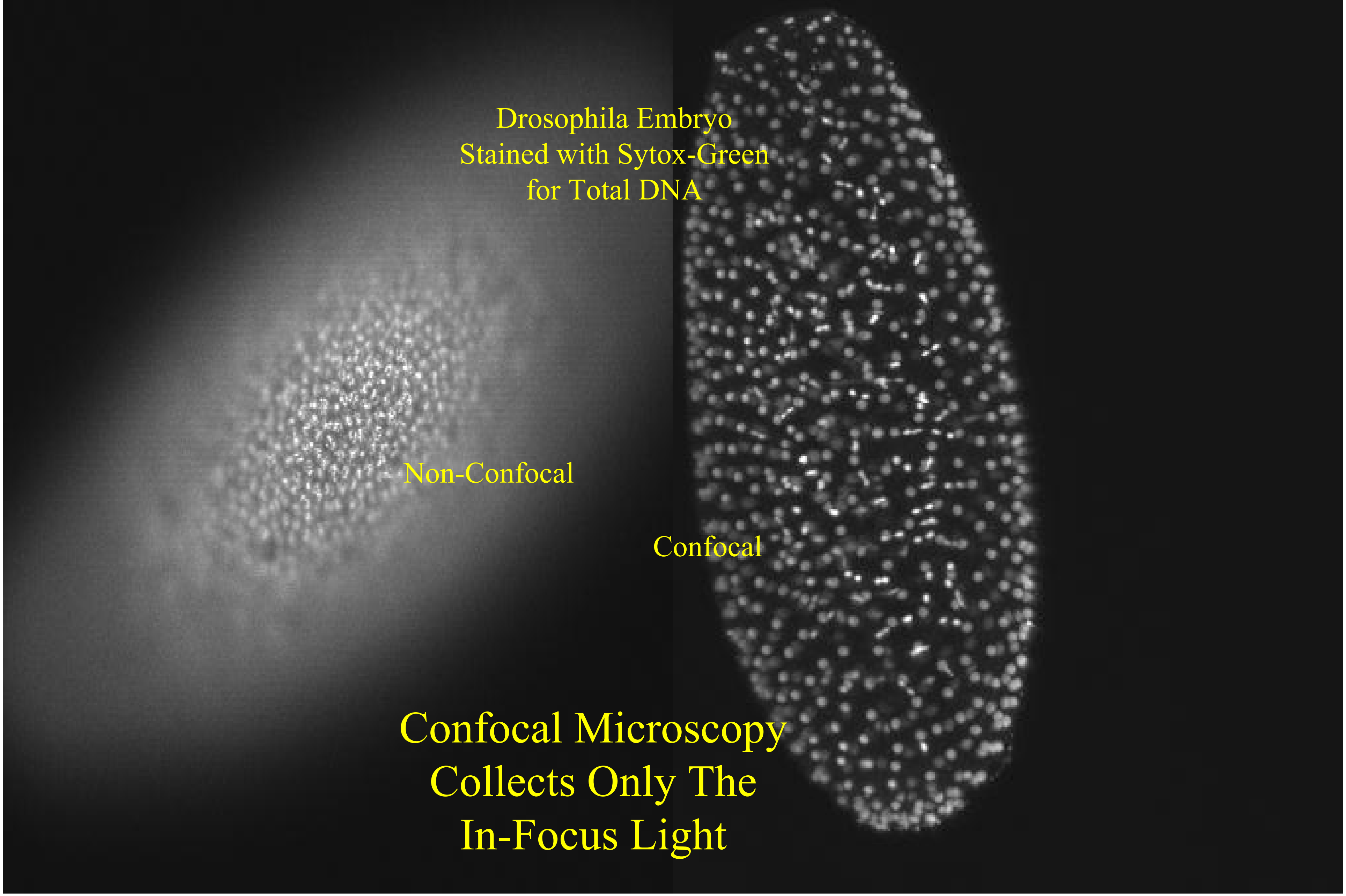
In confocal microscopy a pin hole is placed at the image to block light not focused at that point.

Although the pin hole blocks most of the out-of-focus light, only one in-focus point can be collected at a time.

This means the detector can be a simple photon counting device. A PMT is used.

It also means the illumination must be scanned over the object so an entire image can be collected. A laser is used.

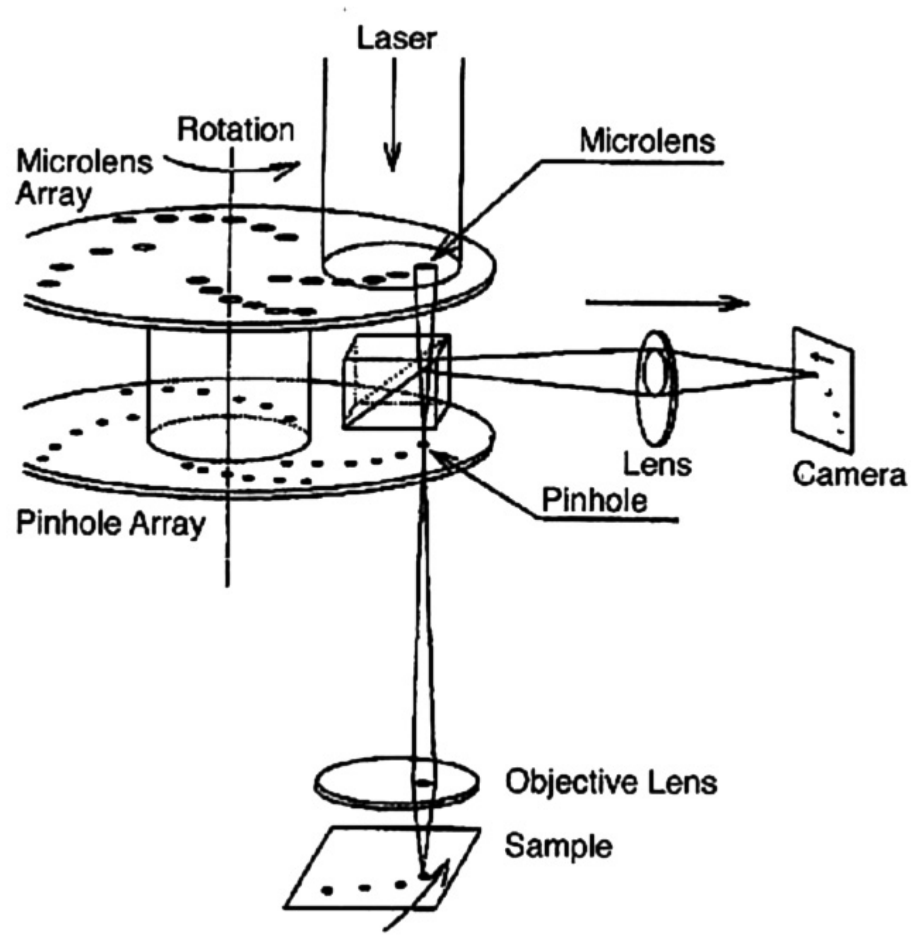
What Does Confocal Give You ?



Confocal Microscopy
Collects Only The
In-Focus Light

Yokogawa's CSU-10 Spinning Disk Confocal System

• The CSU-10 uses a dual Nipkow disk (Paul Nipkow 1884) confocal illumination/imaging mechanism.
• The first disk is an array of ~ 20,000 micro-lenses.
• Collimated excitation illuminates the micro-lens. Each lens focuses the light to a corresponding pin hole on the second disk.
• The objective lens then projects an image of each illuminated pin hole onto the specimen. The disks are rotated together at 1800 rpm allowing the points of light to raster-scan the specimen.
• The fluorescence emission, from each illuminated point on the specimen, is collected by the objective and returned to the second Nipkow disk which now confocalize the light by blocking the out-of-focus components.
• A dichroic mirror, located between the disks, then reflects the fluorescent confocal image to the camera or ocular port.
• The pinhole pattern is designed to capture 12 frames per rotation or 360 frames per second of confocal images.



Do Your Homework !

The goal of the BioImaging Research Group is to manage a successful imaging facility. You can help by having a clear research plan for the imaging component of your experiment. If you have any questions or doubts about your research plan get advice before you begin. For each new project it will be necessary that you submit your concise research plan to the BioImaging group so that we can advise when necessary and keep track of what is being done.

Your research plan should include the following: 1)Cell type to image, 2)How the cells were prepared, 3)How the cells were mounted for imaging, 4)What fluorphors were used, 5)Which objective lens you intend to use, 6)What you hope to see.

It is very important that you have access to a fluorescence microscope, in good working order, with lens and dichroics similar to those you intend to use at our facility. This is the only way in which to insure the biology in your experiment is prepared and mounted properly.

There should be no autofluorescence from the mountant. The coverglass needs to be strongly affixed to the slide so that both can be repeatedly cleaned.

Check the fluorescence staining is bright and that features you intend to image are evident. If you can not see what you intend to image on your fluorescence microscopy there is little chance you will be able to image it on ours.

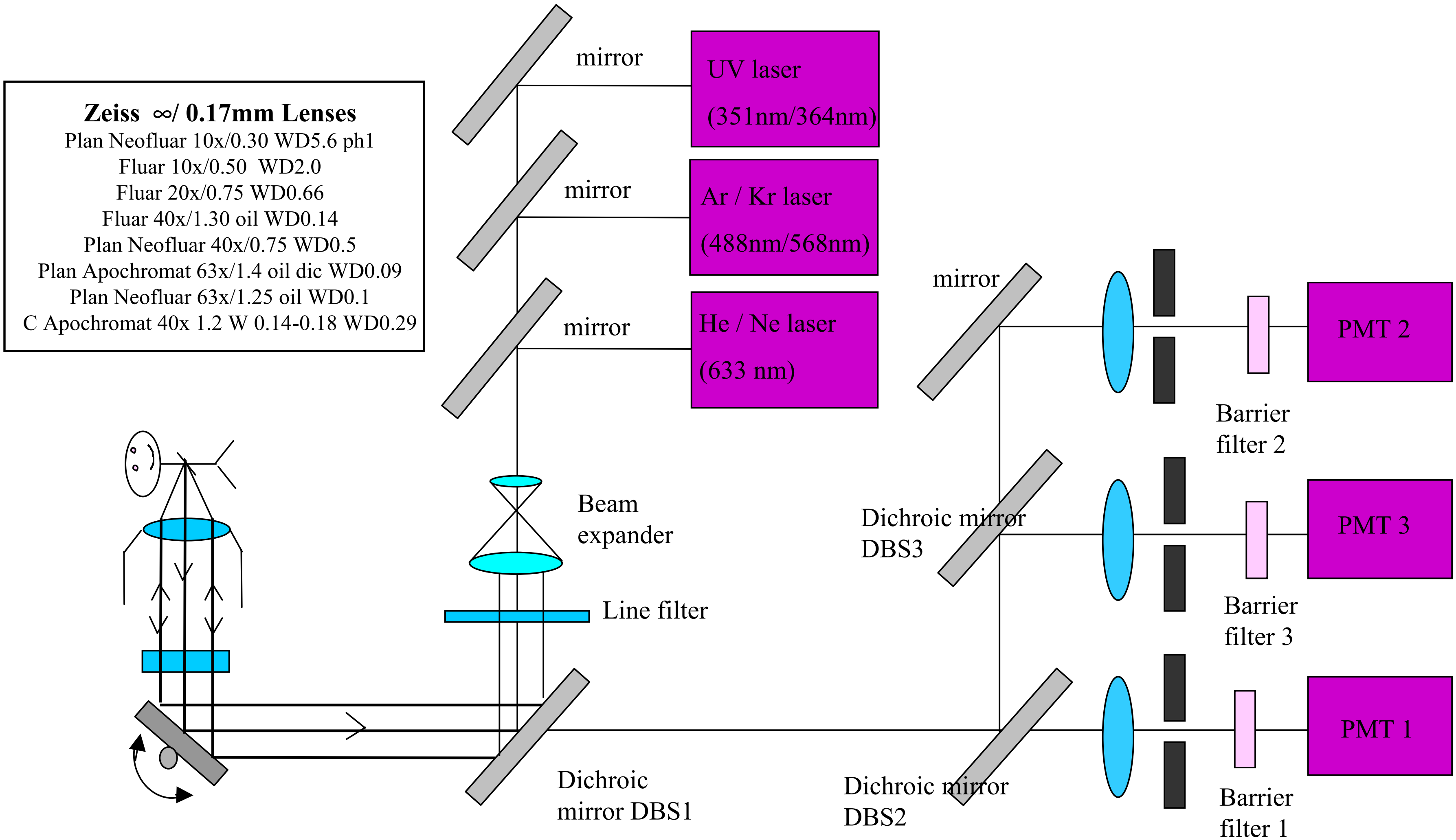
Check the fluorophors you intend to use are compatible with the microscope you intend to use.

If you have any doubts, get advise first !!

Objectives for Fluorescence Imaging

Type of Objective	Image Flatness	Brightfield Observation	Fluorescence Brightness UV	Fluorescence Brightness Blue/Green	Price	Comment
Plan Apochromat	Yes	Best	Medium	High	Highest	Best Brightfield, chromatic aberration free, excellent for UV over 380nm
Fluor	No	Excellent at center	Highest	Highest	High	Recommended for low light fluorescence
Plan Fluor	Yes	Excellent	High	Highest	Medium	Excellent for all fluorescence and brightfield
Plan Achromat	Yes	Excellent	Lowest	Good	Medium	Good fro blue/green excitation, excellent brightfield
E Plan Achromat	Yes	Good	Low	High	Low	Ok for blue/green excitation, good price
Achromat	No	Fair	Low	Fair	Lower	Ok for bright specimens, low budget
E Achromat	No	Fair	Low	Fair	Lowest	Ok for bright specimens, low budget

LSM 410 Optical Configuration



LSM 410 Wavelength Configuration

March 2002

Lasers:
*Omnichrome Series 43
488/568 Kr/Ar
*633 He/ Ne
*Coherent Enterprise II
351/364 UV

Line Selection Filters
D364/8x C
D351/8x C

Filter arrow towards
the microscope object

Mirror Z

R/FL2
BP 590-610 Z
BP 515-565 Z
BP 510-525 Z
BP 510-530 C

PMT 2

DBS3
Mirror Z
FT 560 Z
Q495LP C

R/FL3
BP 515-540 Z
LP 400 C
BP 400-430 C

PMT 3

DBS1

DBS2
Free
FT – 560 Z
FT – 630 Z

R/FL1
LP 515 Z
LP 590 Z
RG 665 Z
BP 575-640 Z
BP 515-565 Z
BP 510-525 Z

PMT 1

FT - 488 / 568 Z
NT - 80/20 Z
FT – 655 Z

FT - 395 Z(new)
84100BS Quad C
empty

Refl / Trans (Quad Dichroic)
<410 / 420-440, 450-480
490+-10 / 500-540
555+-10 / 565-630
640+-10 / >650nm

Barrier Filters 18mm dia
Dichroics 25mmx35mm

{ On order from Chroma: HQ665LP, HQ645/75m, HQ572LP }

Z = Zeiss
C = Chroma

Test Yourself

A list of questions:
The following is a list of questions that cover some of the material from this class. They emphasize essentials in microscopy and have been designed as a knowledge check list. It is the hope that any time you use a microscope you understand these questions, their answers and could explain them to others. It will be expected that you can do this before the practical-confocal microscopy part of this course. I encourage you to come and ask any questions you may have on microscopy.

1) What is refraction and why is a lens made of glass ?
2) What is the function of a lens ?
3) Why is it important to have the correct cover glass thickness for a lens ?
3) What is resolution ?
4) How are numerical aperture (NA), working distance (WD) and resolution related ?
5) What is fluorescence ?
6) What is the function of a dichroic mirror ?
7) Use a diagram to illustrate the essence of epi-fluorescence microscopy.

Bibliography

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